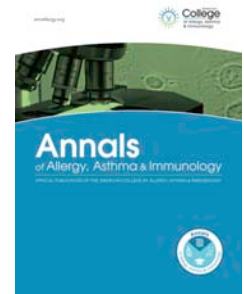


Accepted Manuscript

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PII: S1081-1206(19)30395-3

DOI: <https://doi.org/10.1016/j.anai.2019.05.019>

Reference: ANAI 2921

To appear in: *Annals of Allergy, Asthma and Immunology*

Received Date: 26 February 2019

Revised Date: 15 May 2019

Accepted Date: 23 May 2019

Please cite this article as: Tokunaga Y, Imaoka H, Kaku Y, Kawayama T, Hoshino T, The significance of CD163 expressing macrophages in asthma, *Annals of Allergy, Asthma and Immunology* (2019), doi: <https://doi.org/10.1016/j.anai.2019.05.019>.

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The significance of CD163 expressing macrophages in asthma

Short title: CD163 and macrophages in asthma

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Conflict of Interest; None.

Funding source; This work was supported by GSK Japan Research Grant (H.I.), Allergy Foundation (T.H. and H.I.) and Takeda Science Foundation (Tokyo, Japan) (H.I.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Keywords; CD163, asthma, macrophage, chronic obstructive pulmonary disease, COPD, airway hyper-responsiveness

Abbreviations; AB-PAS: Alcian blue-periodic acid-Schiff, ACh: Acetylcholine, ACO: Asthma-COPD overlap, AHR: Airway hyper-responsiveness, BALF: Bronchoalveolar lavage fluid, COPD: Chronic obstructive pulmonary disease, HDM: House dust mite, HE: Hematoxylin and eosin, ICS: Inhaled corticosteroid, IHC: Immunohistochemistry, KO: Knockout, OCS: Oral corticosteroid, OVA: Ovalbumin, SD: Standard deviation, SEM: Standard error of the mean, WT: Wild-type

Word count; 3152 words

Figures; 6

Tables; 2

The significance of CD163 expressing macrophages in asthma

Introduction

Asthma is characterized clinically by airflow limitation and airway hyper-responsiveness (AHR) induced by chronic lower airways inflammation. The inflammatory process in allergic asthma is initiated by T-helper 2 (Th2) CD4 + cells, which produce a repertoire of cytokines, including IL-4, IL-5, IL-9, and IL-13. These cytokines play a critical role in IgE production, airway eosinophilia, and goblet cell hyperplasia ¹. The histopathologic characteristics of lungs in fatal cases of asthma include severe airway remodeling accompanied by hypertrophy of the smooth muscle, airway wall edema, hyperplasia, and hypertrophy of goblet cells, mucous plugs, and massive pulmonary inflammation ². The asthma mortality rates were maintained at a high level of 0.62 per 100,000 in late 1980s until the widespread use of inhaled corticosteroid (ICS) therapy. Next, the asthma mortality rates reduced to a level of 0.23 per 100,000 in 2000s ³. The burden of asthma continues to be quite high and Japan continues to have the highest asthma-related mortality among developed nations; the age-standardized asthma mortality rate in Japan was 9.34 per million across all ages in the period 2001–2010 ⁴.

Macrophages can be broadly classified into 2 major subtypes: classically activated phenotypes cells (M1) and alternatively activated cells (M2). M1 macrophages, induced by

IFN- γ and lipopolysaccharide (LPS), upregulate the expression of genes involved in pathogen clearance and drive inflammation in response to intracellular pathogens. In contrast, M2 macrophages, induced by IL-4 and IL-13, up-regulate the expression of genes involved in wound healing, clearance of dead and dying cells and tissues, and are involved in anti-inflammatory responses⁵⁻⁷. These two macrophage states mirror the Th1-Th2 polarization of T cells⁸⁻¹⁰. In the lung tissues in asthma patients to protect tissue from injury by microorganisms and to repair the tissue, macrophages appear to be less prone to polarization toward either the M1 or M2 phenotypes¹¹.

Macrophage activation is extremely complex and heterogeneous to be divided into 2 subtypes; recent researchers often use the term “M1-like/M2-like” in the phenotype of macrophage activation. CD163, a scavenger-receptor specifically expressed on macrophages, is known as a marker for tissue repair/protumor/M2-like macrophages^{12, 13}. CD163 is known to be secreted from activated macrophages by shedding with proteases, and the plasma level of soluble CD163 is reportedly increased under inflammatory conditions in several diseases, including in lung diseases¹⁴⁻¹⁶.

Asthma is characterized by chronic airway inflammation and eosinophilic inflammation that are induced by Th2 cell-mediated immune response. The presence of a correlation between M2-like macrophages and Th2 inflammation is well known¹⁷. However, the distribution and roles of CD163 in asthma remains unclear. Therefore, we examined the distribution and roles

of CD163 using human lung specimens and mouse models.

Methods

Patients

We study carefully excluded smokers and ex-smokers and analyzed the nonsmokers to exclude those with chronic obstructive pulmonary disease (COPD). Nine patients diagnosed with asthma had been treated at the Kurume University Hospital (Kurume, Japan) and the Fukuoka National Hospital (Fukuoka, Japan). All patients were diagnosed by physicians. Medical records showed that all patients had histories of transient paroxysmal and repeated wheezes and dyspnea with spontaneous improvement, or use of bronchodilators or oral corticosteroids. These asthma patients were nonsmokers and had died between 1973 and 1999. Their lung tissues were obtained at autopsy examination. As materials for comparison, the normal lung tissues were obtained as controls from 8 nonsmokers (2 men and 6 women; age range, 55–73 years), all of whom underwent surgical resection for lung cancer at the Kurume University Hospital from 2000 to 2005. No significant difference was observed in the ages of patients with fatal asthma and the non-asthma control subjects. However, samples of patients with asthma after 1999 and control samples between 1973 and 1999 could not be obtained from the two hospitals.

Sample collection and all procedures were approved by the ethics committees of the

Kurume University in accordance with the ethical standards of the Declaration of Helsinki of 1975.

Histology

The lung tissues were fixed with 10% formalin and embedded in paraffin wax, as reported previously¹⁸. Paraffin-embedded lung tissues (1–3 in numbers) were obtained from each patient. Sequential sections were made from each paraffin-embedded lung tissue. Sections (4- μ m thick) were serially cut, placed on poly-L-lysine-coated slides, and incubated overnight at 55°C–60°C, as described previously².

Immunohistochemical Staining for Human Subjects

For the blockade of endogenous peroxidase activity, 4- μ m thick deparaffinized sections were incubated with 1% H₂O₂ for 30 min. To detect the macrophages, the tissues were reacted overnight at 4°C with anti-human CD68 (mouse monoclonal, clone PG-M1, Agilent Technol, Santa Clara, CA, USA) and anti-human CD163 (mouse monoclonal, clone 10D6, Leica Biosystems, Nussloch, Germany) antibodies, and control nonimmune mouse IgG (DAKO) was used as the negative control, as described previously¹⁹. Then, the samples were washed extensively and incubated further with appropriate horseradish peroxidase-conjugated secondary antibodies (Nichirei, Tokyo, Japan) for 1 h at the room temperature. After the

removal of unreacted secondary antibodies, the samples were incubated with 3,3'-diaminobenzidine-4HCl (Agilent, Tokyo, Japan)-H₂O₂ solution to visualize immunolabeling. Then, some sections were counterstained with hematoxylin and eosin and mounted with a coverslip, as described previously²⁰.

Quantitative Assessment of CD68+ and CD163+ Macrophages in the Lung Tissue

The quantitative assessment of macrophages was performed as reported previously, with minor modification²¹. Initially, nine square fields in which small-airway inflammation appeared most severe were selected (hot spots)²². The numbers of CD68-positive cells, as macrophages, in the interstitial lung tissues were counted within these nine square fields and expressed in number per millimeter square. The total numbers of macrophages in asthma patients and controls were expressed as mean $\pm$ standard error of the mean (SEM) cells/mm². Then, the CD163+ cells were counted in the same fields as CD68+ cells. Two pathologists examined these sections independently in a blinded manner, without prior knowledge of the patients' clinical status.

Study Design for Mouse Asthma Model

Balb/c wild-type (WT) mice were purchased from Charles River Japan (Yokohama, Japan). CD163-deficient (knockout, KO) mice in the C57BL/6N background were obtained from the

Knockout Mouse Project²³. The CD163KO mice were backcrossed to the Balb/c strain for more than 7 generations. All mice used in this study were of female gender aged 6–8 weeks. The mice were bred under specific pathogen-free conditions and provided with standardized diets at the animal facilities (Kurume University Animal Center). Less than 6 mice were bred in one cage to minimize animal suffering and distress. The method of euthanasia was cervical dislocation or exsanguination in unconscious mice under anesthesia. The Committee for Ethics of Animal Experiments, Kurume University approved all procedures (approval no. 050-058, March 30, 2016), and animal care was provided in accordance with the procedures outlined in the “principle of laboratory animal care” (National Institutes of Health Publication No.86-23, revised 1985). The experimental procedure (Fig 1) has been described previously^{24, 25}. Balb/c CD163 KO mice and control Balb/c WT mice (6–9 mice per group) were treated twice with an intraperitoneal injection of 10 µg sterile chicken ovalbumin (OVA, grade V, Sigma-Aldrich Chemical, St. Louis, MO) emulsified with 4 mg of sterile aluminum hydroxide (Alu-Gel-S Suspension, Serva Electrophoresis GmbH, Heidelberg, Germany) in a total volume of 200 µL. The injections were administered at days 0 and 5. These mice were challenged for 20 min with 0.9% saline (Group 1) or with 5% OVA in 0.9% saline (Group 2) administered via the airways using an ultrasonic nebulizer (Omron NE-U07, Tokyo, Japan) in a closed box. We performed histological analysis to find bronchoalveolar lavage fluid (BALF) and AHR in all groups, as reported previously^{24, 25}. We conducted the experiments

twice each and combined the results.

Histological Analysis in Mice

For the histological analysis, the mice were euthanized via intraperitoneal injection of pentobarbital sodium (2.5–5 mg per mouse). After the thorax was opened, the trachea was dissected free from the underlying soft tissues, and a 0.8-mm tube was inserted through a small incision in the trachea. The lung tissues were immediately fixed by intratracheal instillation of 10% buffered formalin (pH 7.40) for 15–20 min at a constant pressure of 25-cm H₂O. After gross examination, the extracted tissues were placed into 10% buffered formalin and further fixed for at least 24 h. Sections (4-μm thick) were cut from paraffin-embedded tissues, placed on poly-l-lysine-coated slides, and then incubated overnight at 55°C–60°C. The deparaffinized sections were stained with hematoxylin and eosin and alcian blue-periodic acid-Schiff (AB-PAS), as described previously^{24, 26}.

Immunohistochemical Staining for Mice

We performed immunohistochemical staining of Iba1 in lung macrophages and CD163 as M2-like macrophages in the lung of mice. Anti-mouse Iba1 (rabbit polyclonal, WAKO, Tokyo, Japan) and anti-mouse CD163 (rabbit polyclonal, CosmoBio, Tokyo, Japan) antibodies were used as the first antibody for immunohistochemistry (IHC), and IHC were

performed as described above.

Analysis of BALF Obtained from Mice

The trachea was inserted with a tubing adaptor, and the lungs were washed thrice with 3 mL saline. Aliquots of the cells were centrifuged onto glass slides, dried in air, and stained with May–Grunwald–Giemsa stain. The total cell and the differential cell counts were obtained from cytospin preparations, dried in air, and stained with May–Grunwald–Giemsa stain. The cell populations were counted, and the absolute number of cell populations were then calculated, as reported previously^{24, 25}. The supernatants of BALF was kept at -30°C for measurements.

Measurement of IL-4, -5, -13, Eotaxin, and IFN- γ in the Supernatants of BALF

The levels of IL-4, -5, -13, eotaxin, and IFN- γ in the supernatants of BALF were measured using commercially available ELISA Kits (ThermoFisher, Waltham, Massachusetts, USA).

Assessment of AHR

AHR to aerosolized acetylcholine (ACh; Sigma-Aldrich Japan, Tokyo, Japan) was tested 24 h after OVA or saline challenge, as described previously^{24, 25}. Briefly, under mechanical ventilation (150 breaths/min, tidal volume 10 mL/kg, and positive end-expiratory pressure 2

cmH₂O) (Buxco FinePointe RC; Data Science International, MN) after anesthetization and intratracheal intubation via tracheotomy, mean airway resistance of mice was measured automatically after inhalation of 0.9% saline at the baseline, followed by increasing doses of aerosolized ACh (doubling doses from 0 to 160 mg/mL in 20 µL) via a nebulizer (inhalation for 30 s and response for 3 min)²⁵.

Statistical Analysis

All data were expressed as means ± SEM. Differences between 2 and 3 or more groups were compared by Tukey–Kramer test and the analysis of variance with the Tukey Honestly Significant Difference Test, respectively. Differences at $P < 0.05$ were considered to be statistically significant. JMP 12.2.0 (SAS Institute Japan, Tokyo, Japan) was used for statistical analysis.

Results

Clinical Findings

All 9 patients with fatal asthma were nonsmokers, and none had COPD. Their ages ranged from 5 to 79 years (Table 1). Five of the 9 patients died within 24 h of the onset of asthma attack. The duration of disease in these patients varied widely from 2 to 50 years; 7 patients had asthma for >6 years. Six patients had been treated with systemic corticosteroid, and no

patient received ICS. Three patients died without treatment with systemic corticosteroid or ICS (Table 2).

Increased Number of CD68+ and CD163+ Macrophages in Patients with Fatal Asthma

The representative examples of the histology of the lung tissues obtained from 2 patients with fatal asthma are shown (Fig 2A). The control subject was a 48-year-old man. Case 1 patient was a 5-year-old boy (Patient #1 in Table 1), and Case 2 was a 32-year-old man (Patient #2 in Table 1). Immunostaining to detect CD68 and CD163 was successful in 40-year-old specimens of formalin-preserved lung tissues. We performed heat-induced and proteolysis-induced antigen retrieval as described previously¹⁹. This indicated that CD68 and CD163 antigens on the macrophage surface were intact in the formalin-preserved tissues. Immunostaining for CD68 was performed using pathological sections to examine the distribution of lung macrophages. The number of CD68+ macrophages were increased in patients with fatal asthma. It is well known that CD163 is specifically expressed on macrophages and are useful as M2-like macrophage markers. Therefore, we performed immunostaining of CD163 using serial sections. Similar to the number of CD68+ macrophages, the numbers of CD163+ macrophages were significantly increased in the patients with fatal asthma as compared with that in the non-asthma control subjects (Fig 2B). There were no significant differences in the background and cell counts between males and

females in the control group.

CD163⁺ Cells are increased in the Lungs of OVA/OVA-WT Mice

Immunohistochemical staining of Iba1 revealed that the numbers of Iba1⁺ cells were increased in the lungs of OVA-sensitized and OVA-challenged (OVA/OVA-) WT mice as compare with that those in the lungs of OVA-sensitized and saline-challenged (OVA/saline-) WT mice. We performed immunostaining of CD163 using serial sections. Similar to the numbers of Iba1⁺ cells, the numbers of CD163⁺ cells increased in OVA/OVA-WT mice as compare with that in OVA/saline-WT mice (Fig 3).

Decreased Airway Inflammation in CD163 KO Mice Mouse Asthma Model

Histological examination by hematoxylin eosin and alcian blue-periodic acid-Schiff staining revealed decreased airway inflammation and mucous cell metaplasia in the lungs of OVA/OVA-CD163 KO mice compared with the lungs of OVA/OVA-WT mice. In contrast, airway inflammation and mucous cell metaplasia were not observed in the lungs of OVA/saline-CD163 KO mice and WT mice (Fig 4A and B). The cells in BALF with May-Grunwald-Giemsa stain showed decreased eosinophils in OVA/OVA-CD163 KO mice as compared to that in OVA/OVA-WT mice (Fig 4C). BALF analysis revealed that the number (mean $\pm$ SEM, $\times 10^4$ cells/mL) of total cells in OVA/OVA-CD163KO mice was significantly

lower than that in OVA/OVA-WT mice. The number of eosinophils, lymphocyte, neutrophils and macrophages in OVA/OVA-CD163KO mice was significantly lower than that in OVA/OVA-WT mice (Fig 4D).

Decreased Levels of IFN- γ and IL-5 in the BALF of OVA/OVA-CD163KO Mice

We analyzed the protein levels of IL-4, IL-5, IL-13, IFN- γ , and eotaxin in the BALF. The concentrations of IFN- γ and IL-5 was significantly decreased in the BALF of OVA/OVA-CD163KO mice as compared to that in OVA/OVA-WT mice. In contrast, no significant difference was noted in the concentrations of IL-4, IL-13, and eotaxin between OVA/OVA-CD163KO mice and OVA/OVA-WT mice (Fig 5).

AHR Was Suppressed in OVA/OVA-CD163KO Mice

We investigated AHR in OVA/OVA-CD163KO and WT mice on day 19. We found that AHR was increased in OVA/OVA-WT mice as compared to that in OVA/saline-WT mice. In contrast, AHR was suppressed in OVA/OVA-CD163KO mice as compared to that in OVA/OVA-WT mice (Fig 6).

Discussion

M2-like macrophages are associated with Th2 inflammation, and CD163 is known as a

marker for M2-like macrophage¹⁷. In the present study, CD163 was strongly expressed on macrophages in the lungs of all patients with fatal asthma. In the mouse models of asthma, AHR and the numbers of total cells and eosinophils in the BALF were significantly decreased in the OVA/OVA-CD163KO mice when compared with that in the control WT mice.

Several studies have examined CD163 in asthmatic patients. Sputum macrophages from mild and moderate asthma patients expressed less cell-surface CD163 than macrophages from healthy individuals²⁷. On the other hand, the concentration of sputum-soluble CD163 (sCD163) was significantly greater in mild asthma patients than in healthy controls^{16,28}, and the sputum sCD163 level was significantly greater in patients with severe asthma as compared to that in patients with mild/moderate asthma¹⁶. Our present results showed that patients with fatal asthma expressed more CD163-positive macrophages in the lung tissues. It has been a long time since we collected lung tissues from patients with fatal asthma. Presently, asthma patients rarely die from asthma attack because of the widespread awareness of the use of ICS; therefore, it is difficult to collect specimens from the lungs of patients with fatal asthma now. If possible, it is desirable to examine fresh lung tissues in the future. In this study, we examined only the lungs of patients with fatal asthma, and it is desirable to examine the lungs of patients with mild or moderate asthma in the future.

Regarding animal experimentation, in previous researches, the total cells and eosinophils in BALF were significantly increased in HDM and Der p1-challenged-CD163-deficient mice,

and the lung histology similarly revealed an increase in the peri-bronchial inflammatory cell infiltrates in HDM- and Der p1-challenged CD163-deficient mice as compared to that in WT mice²⁹. In contrast, our present results showed that the numbers of total cells and eosinophils in the BALF were significantly decreased in OVA/OVA-CD163KO Balb/c mice. On the other hand, neither HDM-challenged CD163-deficient mice nor WT mice developed methacholine-induced increases in AHR, which likely reflects the C57BL/6 genetic background²⁹. In the present study, AHR was increased in OVA/OVA-WT Balb/c mice as compared to that in OVA/saline-WT Balb/c mice, and it was suppressed in OVA/OVA-CD163KO Balb/c mice as compared to that in OVA/OVA-WT Balb/c mice. This difference may be attributed to the difference in the mice background or antigen used to establish allergy mouse model. Regarding AHR, OVA-sensitized and challenged model may reflect the asthmatic conditions more accurately in Balb/c mice.

In this study, the number of total cells in BALF in OVA/Saline-CD163KO mice was significantly lower than that in OVA/Saline-WT mice. CD163KO mice are complete knockouts of the CD163 gene lack CD163 expression on macrophages. Therefore, the number of total cells, particularly macrophages, may not have increased in OVA/Saline-CD163KO mice. Further analysis is needed to test this possibility.

M2-like macrophages are conventionally considered to display anti-inflammatory properties; recently, they were subdivided further into several subtypes. Some subtypes of

M2-like macrophages are believed to be activated by Th2 cytokines (IL-4 and IL-13) and known to induce allergic immune responses, such as eosinophilic inflammation^{17, 30}. Previous studies have found that M2-like macrophages correlate significantly with the percent of eosinophils in BALF of HDM-induced asthma model³¹. The present study demonstrated that the numbers of eosinophils and IL-5 in the BALF are significantly increased in OVA/OVA-WT mice. However, they are decreased in OVA/OVA-CD163KO mice. A previous study showed that the intranasal administration of anti-IL-5 antibody inhibited the development of eosinophilic lung inflammation and AHR in OVA-sensitized/challenged Balb/c mice³². The reduction of IL-5 may suppress eosinophilic inflammation and AHR in OVA-OVA CD163KO mice. IFN- γ in the BALF are significantly increased in OVA/OVA-WT mice and decreased in OVA/OVA-CD163KO mice. A previous study showed that the IFN- γ expression in the lungs and AHR was induced in Balb/c mice by the transfer of activated eosinophils and that IFN- γ -deficient eosinophils or eosinophils treated with a blocking anti-IFN- γ receptor antibody failed to induce AHR in mice³³. The reduction of IFN- γ may also contribute to suppress AHR in OVA-OVA-CD163KO mice.

Presently, asthma-COPD overlap (ACO) is gained increasing recognition³⁴. Previously, we reported that CD163-positive macrophages are expressed on alveolar macrophages in the lungs of severe COPD patients²⁰. This common feature of asthma and COPD patients suggests that CD163 may be involved in the development of ACO.

Our study had several limitations. First, our study did not investigate the correlation between the number of CD163+ macrophages and AHR in patients with mild or moderate asthma. Second, the incidence and severity of asthma has been reported to be greater in women than in men³⁵ and in female mice compared with male mice^{36, 37}. We performed experiments investigating airway inflammation in the murine asthma model using male and female mice. However, male mice did not exhibit airway inflammation via using our methods^{24, 25}. Male mice have been shown to produce lower amounts of Th2 cytokines and specific IgE, and have fewer lung lymphocytes after OVA sensitization and challenge in the asthma model³⁷. Castrated mice have been found to have increased OVA-induced eosinophil and lymphocyte infiltration in BALF³⁸. Therefore, we included only female mice in the present study. Third, no significant difference was noted in the protein levels of IL-10 in BALF between OVA/OVA-CD163KO mice and OVA/OVA-WT mice, and IL-33 was not detected in BALF using ELISA (data not shown), although a previous study reported that IL-33 released from airway epithelial cells after antigen challenge can modulate M2 macrophage polarization through ST2³⁹. This discrepancy may be due to differences between in vivo and in vitro studies.

The present study showed that the numbers of CD163-positive macrophages were significantly increased in the lungs of patients with fatal asthma. Furthermore, AHR was suppressed and the number of total cells, eosinophils, and neutrophils of BALF were

305 decreased in OVA/OVA-CD163 KO mice as compared to that in OVA/OVA-WT mice. Our
306 result suggests that the inhibition of CD163 can improve eosinophilic inflammation and
307 suppress AHR. Thus, CD163 or CD163-associated macrophage activation may play
308 important roles in airway inflammation and AHR in asthma.

References

1. Izuhara K, Ohta S, Shiraishi H, et al. The mechanism of mucus production in bronchial asthma. *Curr Med Chem*. 2009;16:2867-2875.
2. Oda H, Kawayama T, Imaoka H, et al. Interleukin-18 expression, CD8(+) T cells, and eosinophils in lungs of nonsmokers with fatal asthma. *Ann Allergy Asthma Immunol*. 2014;112:23-28 e1.
3. Wijesinghe M, Weatherall M, Perrin K, Crane J, Beasley R. International trends in asthma mortality rates in the 5- to 34-year age group: a call for closer surveillance. *Chest*. 2009;135:1045-1049.
4. GBD 2015 Chronic Respiratory Disease Collaborators. Global, regional, and national deaths, prevalence, disability-adjusted life years, and years lived with disability for chronic obstructive pulmonary disease and asthma, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet Respir Med*. 2017;5:691-706.
5. Liu C, Li Y, Yu J, et al. Targeting the shift from M1 to M2 macrophages in experimental autoimmune encephalomyelitis mice treated with fasudil. *PLoS One*. 2013;8:e54841.
6. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. *The Journal of clinical investigation*. 2012;122:787-795.
7. Spence S, Fitzsimons A, Boyd CR, et al. Suppressors of cytokine signaling 2 and 3

- 20 diametrically control macrophage polarization. *Immunity*. 2013;38:66–78.
- 21 8. Epelman S, Lavine KJ, Randolph GJ. Origin and functions of tissue macrophages.
22 *Immunity*. 2014;41:21–35.
- 23 9. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte
24 subsets: cancer as a paradigm. *Nature immunology*. 2010;11:889–896.
- 25 10. Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M. Macrophage plasticity
26 and polarization in tissue repair and remodelling. *The Journal of pathology*. 2013;229:176–
27 185.
- 28 11. Moreira AP, Hogaboam CM. Macrophages in allergic asthma: fine-tuning their pro-
29 and anti-inflammatory actions for disease resolution. *J Interferon Cytokine Res*. 2011;31:485-
30 491.
- 31 12. Bai J, Adriani G, Dang TM, et al. Contact-dependent carcinoma aggregate dispersion
32 by M2a macrophages via ICAM-1 and beta2 integrin interactions. *Oncotarget*. 2015;6:25295-
33 25307.
- 34 13. Colin S, Chinetti-Gbaguidi G, Staels B. Macrophage phenotypes in atherosclerosis.
35 *Immunol Rev*. 2014;262:153-166.
- 36 14. Kowal K, Silver R, Slawinska E, Bielecki M, Chyczewski L, Kowal-Bielecka O.
37 CD163 and its role in inflammation. *Folia Histochem Cytobiol*. 2011;49:365-374.
- 38 15. Suzuki Y, Shirai M, Asada K, et al. Utility of Macrophage-activated Marker CD163

- for Diagnosis and Prognosis in Pulmonary Tuberculosis. *Ann Am Thorac Soc*. 2017;14:57-64.
16. Zhi Y, Gao P, Li W, et al. Soluble CD163 Levels and CD163+CD14+ Monocyte/Macrophage Counts in Patients with Asthma. *Iran J Immunol*. 2018;15:239-245.
17. Jiang Z, Zhu L. Update on the role of alternatively activated macrophages in asthma. *J Asthma Allergy*. 2016;9:101-107.
18. Kitasato Y, Hoshino T, Okamoto M, et al. Enhanced expression of interleukin-18 and its receptor in idiopathic pulmonary fibrosis. *Am J Respir Cell Mol Biol*. 2004;31:619-625.
19. Nakagawa T, Ohnishi K, Kosaki Y, et al. Optimum immunohistochemical procedures for analysis of macrophages in human and mouse formalin fixed paraffin-embedded tissue samples. *J Clin Exp Hematop*. 2017;57:31-36.
20. Kaku Y, Imaoka H, Morimatsu Y, et al. Overexpression of CD163, CD204 and CD206 on alveolar macrophages in the lungs of patients with severe chronic obstructive pulmonary disease. *PLoS One*. 2014;9:e87400.
21. Imaoka H, Hoshino T, Takei S, et al. Interleukin-18 production and pulmonary function in COPD. *Eur Respir J*. 2008;31:287-297.
22. Shui R, Yu B, Bi R, Yang F, Yang W. An interobserver reproducibility analysis of ki67 visual assessment in breast cancer. *PLoS One*. 2015;10:e0125131.
23. Shiraishi D, Fujiwara Y, Horlad H, et al. CD163 Is Required for protumoral activation of macrophages in human and murine sarcoma. *Cancer Res*. 2018;78:3255-3266.

- 58 24. Ichiki H, Hoshino T, Kinoshita T, et al. Thioredoxin suppresses airway
59 hyperresponsiveness and airway inflammation in asthma. *Biochem Biophys Res Commun.*
60 2005;334:1141-1148.
- 61 25. Sawada M, Kawayama T, Imaoka H, et al. IL-18 induces airway hyperresponsiveness
62 and pulmonary inflammation via CD4⁺ T cell and IL-13. *PLoS One.* 2013;8:e54623.
- 63 26. Imaoka H, Hoshino T, Okamoto M, et al. Endogenous and exogenous thioredoxin 1
64 prevents goblet cell hyperplasia in a chronic antigen exposure asthma model. *Allergol Int.*
65 2009;58:403-410.
- 66 27. Staples KJ, Hinks TS, Ward JA, Gunn V, Smith C, Djukanovic R. Phenotypic
67 characterization of lung macrophages in asthmatic patients: overexpression of CCL17. *J*
68 *Allergy Clin Immunol.* 2012;130:1404-1412 e7.
- 69 28. Kowal K, Moniuszko M, Bodzenta-Lukaszyk A. The effect of inhaled corticosteroids
70 on the concentration of soluble CD163 in induced sputum of allergic asthma patients. *J*
71 *Investig Allergol Clin Immunol.* 2014;24:49-55.
- 72 29. Dai C, Yao X, Gordon EM, et al. A CCL24-dependent pathway augments eosinophilic
73 airway inflammation in house dust mite-challenged Cd163(-/-) mice. *Mucosal Immunol.*
74 2016;9:702-717.
- 75 30. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and
76 polarization. *Front Biosci.* 2008;13:453-461.

- 77 31. Draijer C, Robbe P, Boorsma CE, Hylkema MN, Melgert BN. Characterization of
78 macrophage phenotypes in three murine models of house-dust-mite-induced asthma.
79 *Mediators Inflamm.* 2013;2013:632049.
- 80 32. Hamelmann E, Cieslewicz G, Schwarze J, et al. Anti-interleukin 5 but not anti-IgE
81 prevents airway inflammation and airway hyperresponsiveness. *Am J Respir Crit Care Med.*
82 1999;160:934-941.
- 83 33. Kanda A, Driss V, Hornez N, et al. Eosinophil-derived IFN-gamma induces airway
84 hyperresponsiveness and lung inflammation in the absence of lymphocytes. *J Allergy Clin*
85 *Immunol.* 2009;124:573-582, 82 e1-9.
- 86 34. Global Initiative for Asthma. Global strategy for asthma management and prevention.
87 2018. Available from <https://ginasthma.org/gina-reports/> Cited.
- 88 35. Hansen S, Probst-Hensch N, Keidel D, et al. Gender differences in adult-onset
89 asthma: results from the Swiss SAPALDIA cohort study. *Eur. Respir. J.* 2015;46:1011–1020.
- 90 36. Blacque`re MJ, Hylkema MN, Postma DS, Geerlings M, Timens W, Melgert BN.
91 Airway inflammation and remodeling in two mouse models of asthma: comparison of males
92 and females. *Int. Arch. Allergy Immunol.* 2010;153:173–181.
- 93 37. Melgert BN, Postma DS, Kuipers I, et al. Female mice are more susceptible to the
94 development of allergic airway inflammation than male mice. *Clin. Exp. Allergy.*
95 2005;35:1496–1503.

- 96 38. Hayashi T, Adachi Y, Hasegawa K, Morimoto M. Less sensitivity for late airway
97 inflammation in males than females in BALB/c mice. *Scand. J. Immunol.* 2003;57:562–567.
- 98 39. Nabe T, Wakamori H, Yano C, et al. Production of interleukin (IL)-33 in the lungs
99 during multiple antigen challenge-induced airway inflammation in mice, and its modulation
100 by a glucocorticoid. *Eur J Pharmacol.* 2015;757:34-41.

Tables

Table 1. Characteristics of 9 nonsmokers with fatal asthma

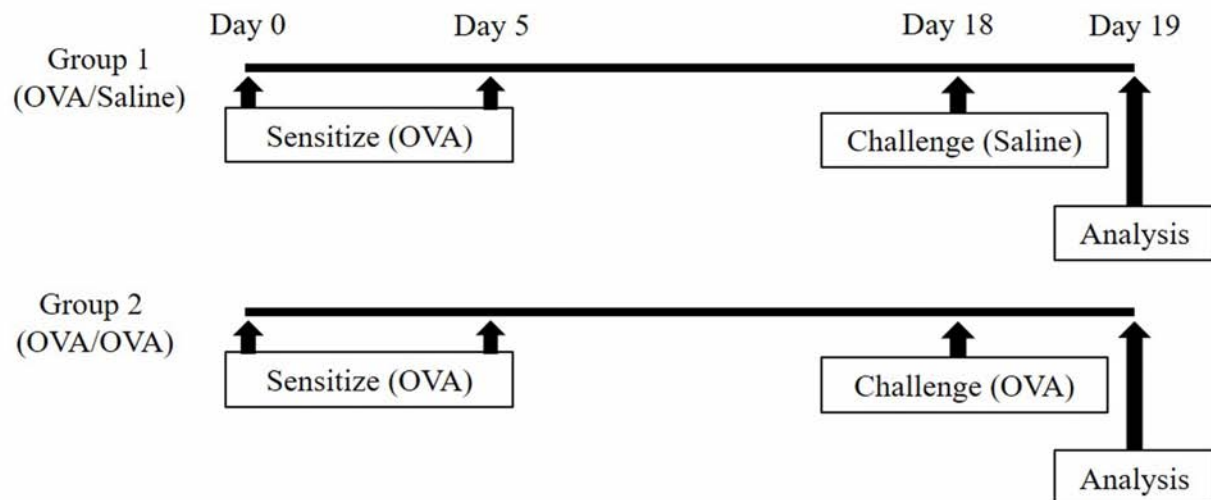
Patient number	Age	Sex	Year at autopsy	Therapy						Duration from onset
				OCS	ICS	β 2-agonist	Theophylline	LTRA	Ventilation	
1	5	M	1977	-	-	-	-	-	+	36 h
2	32	M	1973	+	-	-	+	-	-	75 min
3	67	M	1980	-	-	-	-	-	-	5 h
4	44	M	1981	+	-	-	-	-	-	< 24 h
5	75	M	1982	+	-	-	-	-	-	20 min
6	57	M	1986	+	-	-	-	-	-	unknown
7	16	F	1984	-	-	+	+	-	-	6 h
8	79	F	1986	+	-	-	-	-	-	7 days
9	67	F	1999	+	-	-	-	-	+	26 days

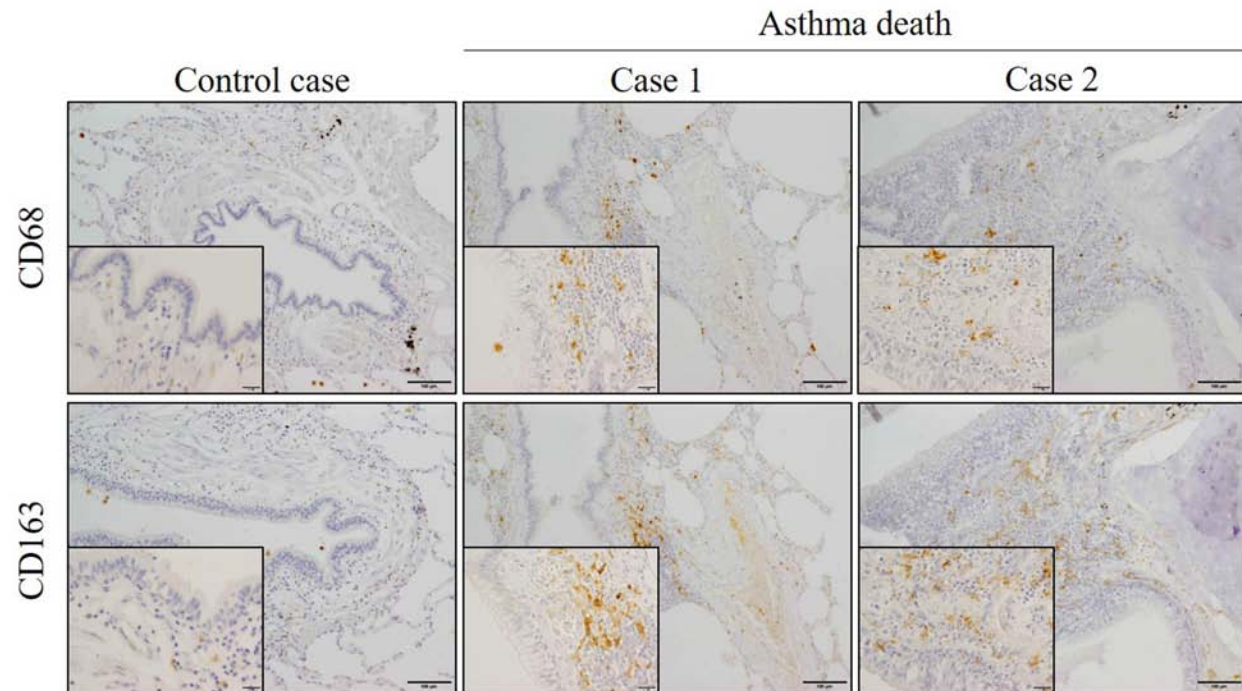
Abbreviations: ICS, inhaled corticosteroid; LTRA, leukotriene receptor antagonist; M, male; OCS, oral corticosteroid.

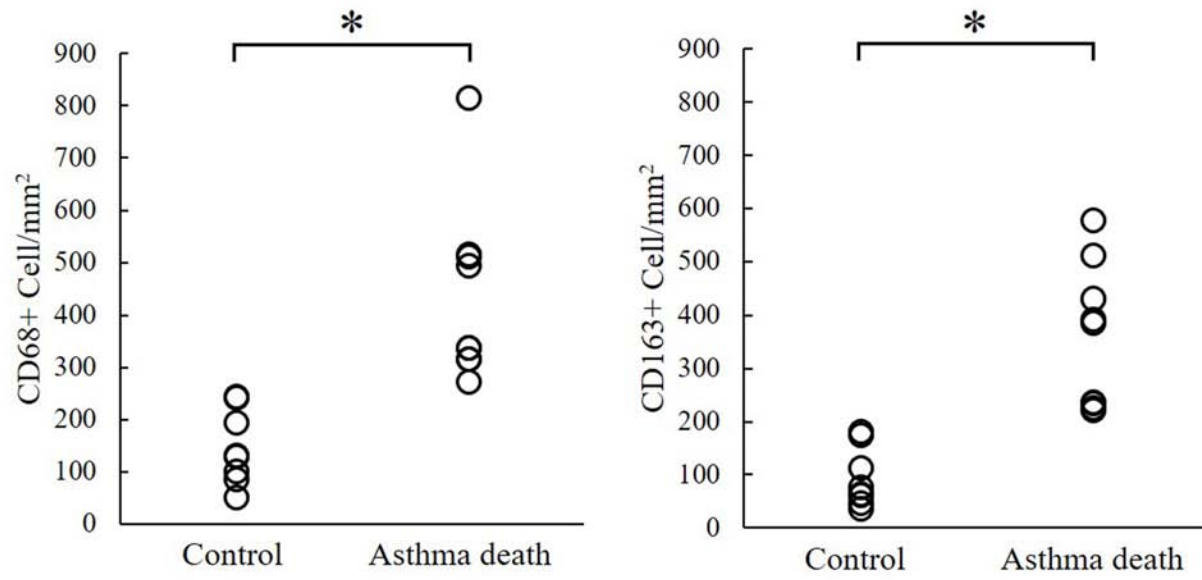
Table 2. Characteristics of patients with fatal asthma and control patients

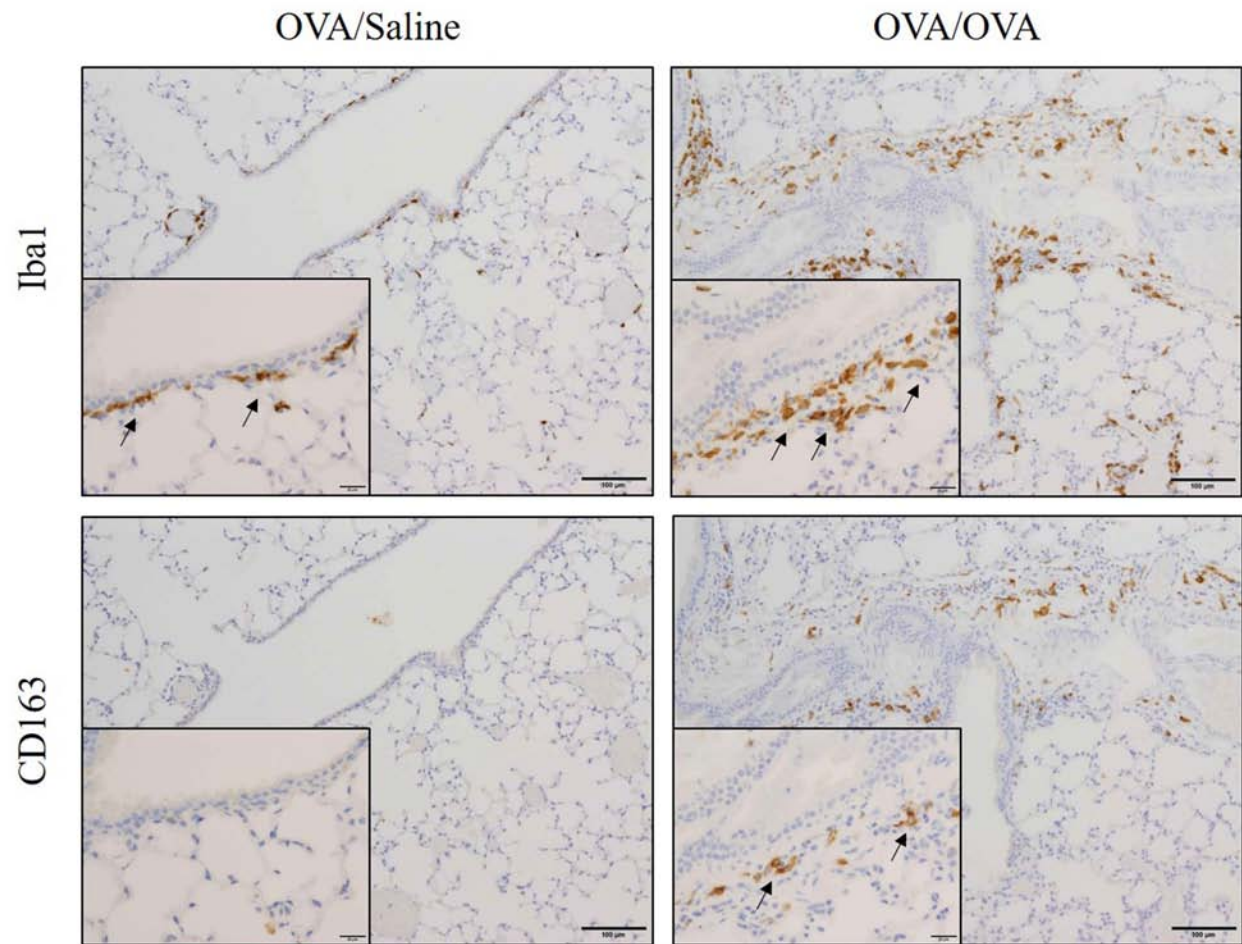
	Control	Asthma death
Patients (male/female), n	8 (2/6)	9 (6/3)
Age (y), mean±SD	62.6 ± 7.9	49.11±25.0
Body mass index (kg/m²), mean ± SD	24.5 ± 3.1	21.49±2.49
VC (% predicted), mean ± SD	122.3 ± 13.3	ND
FEV₁ (% predicted), mean ± SD	113.0 ± 11.9	ND
FEV₁/FVC (%), mean ± SD	75.1 ± 5.8	ND

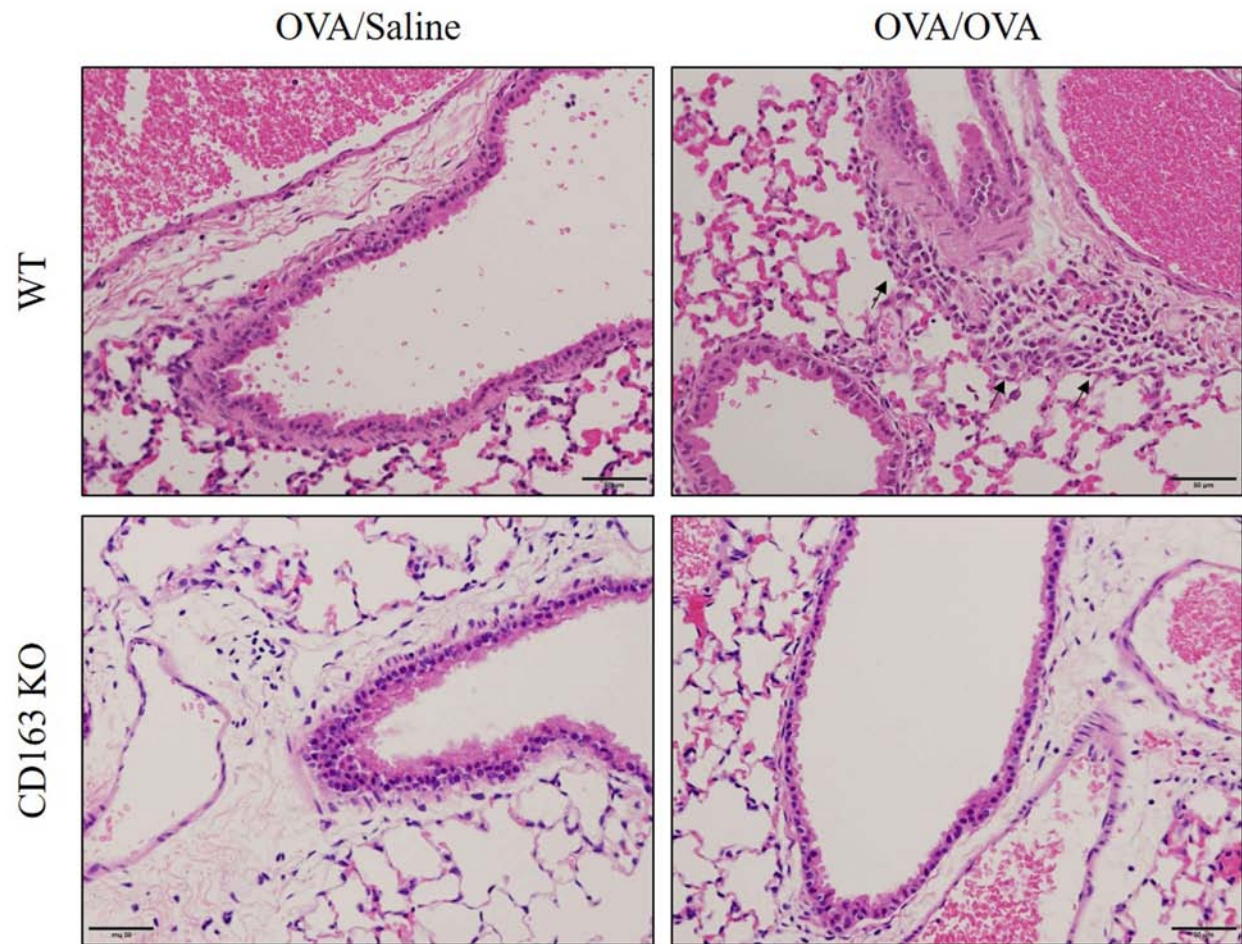
Abbreviations: FEV₁, forced expiration in 1 second; FVC, forced vital capacity; ND, not done; SD, standard deviation.

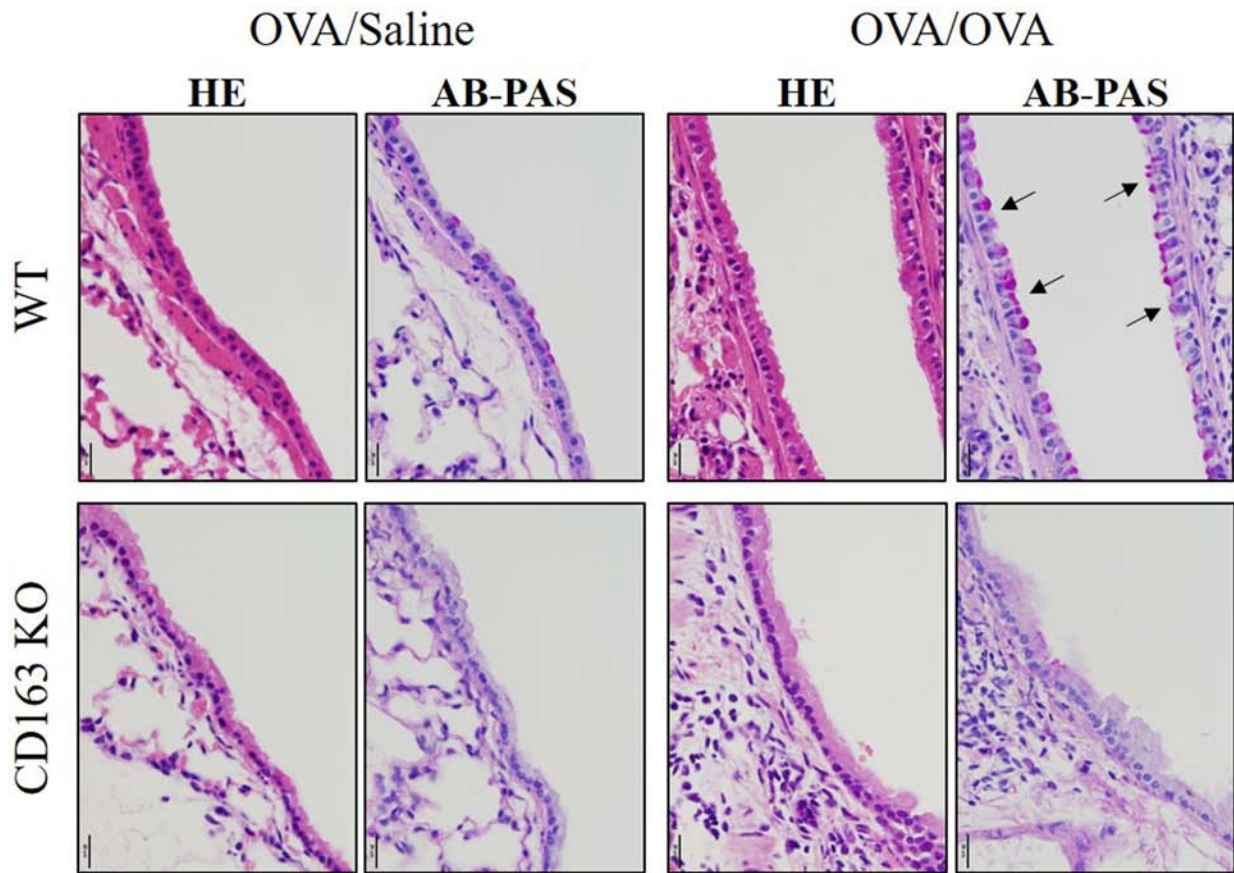


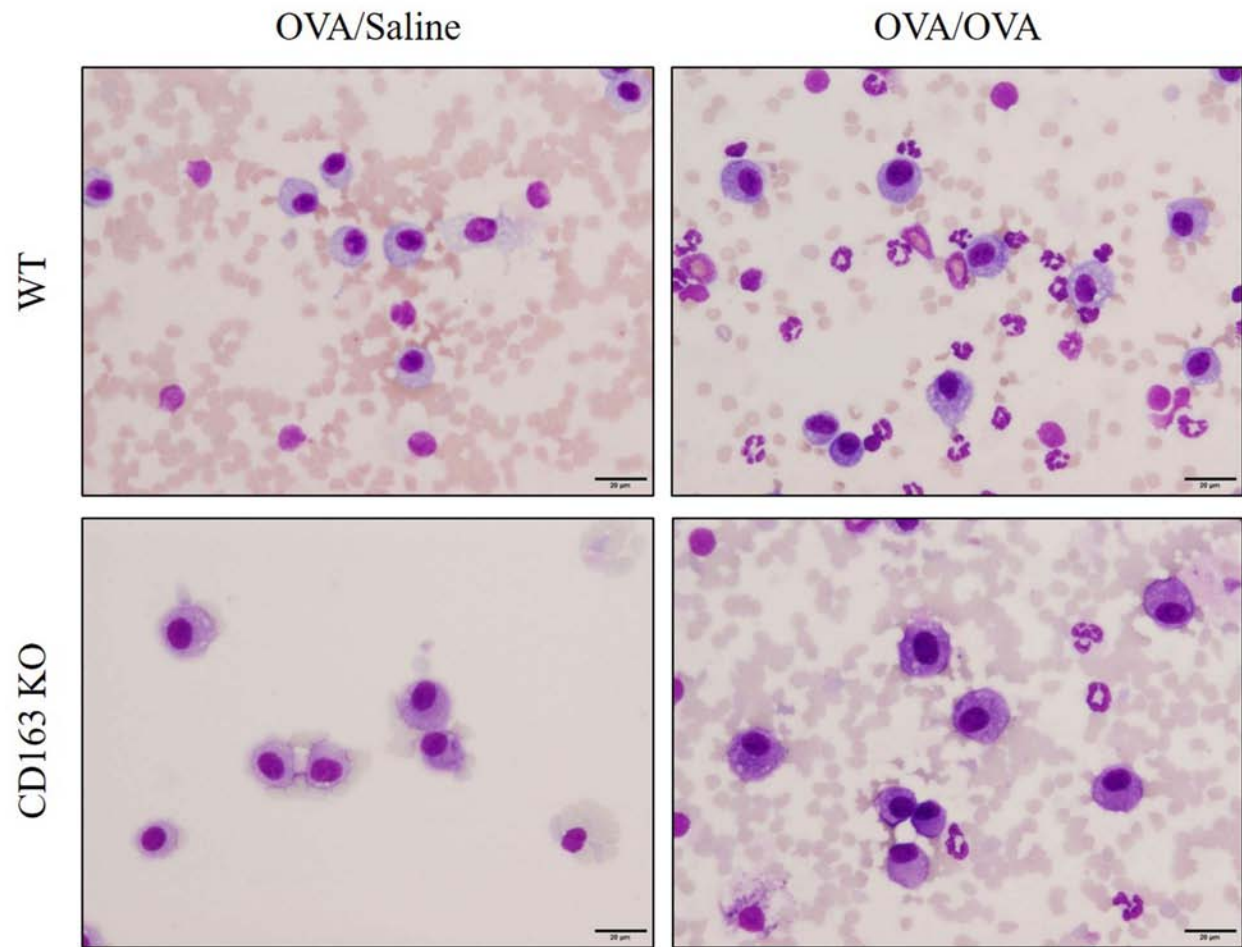


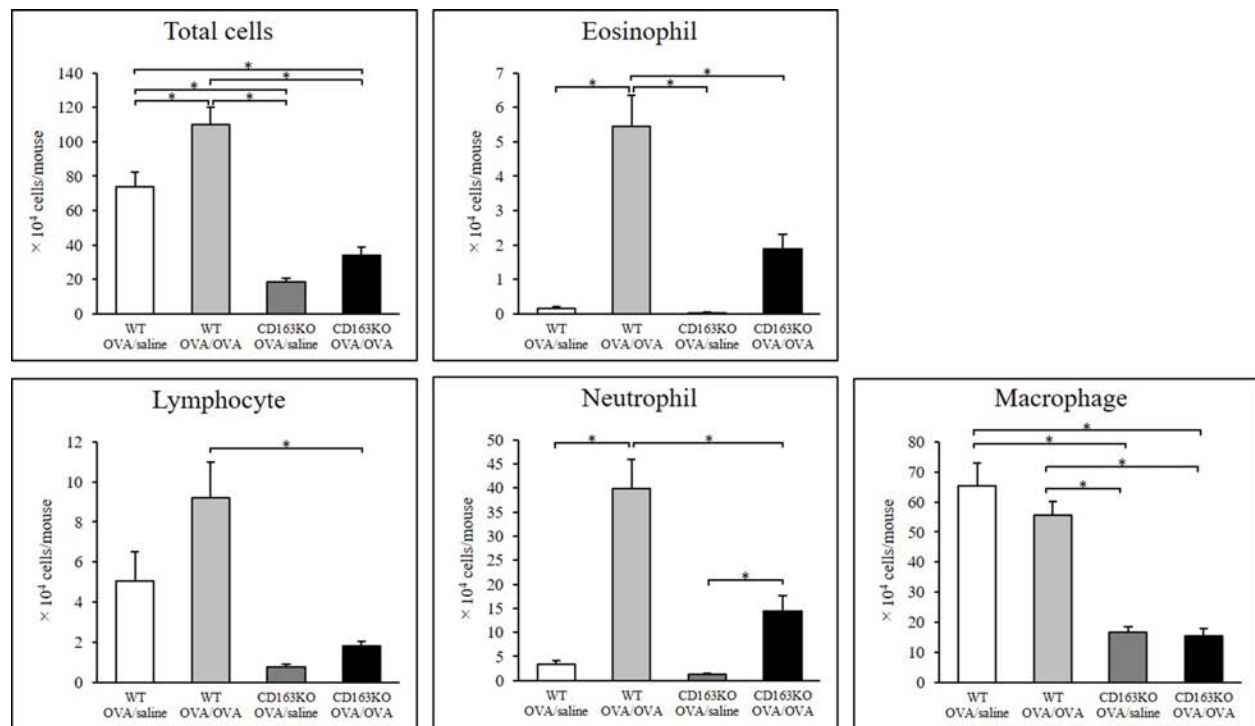


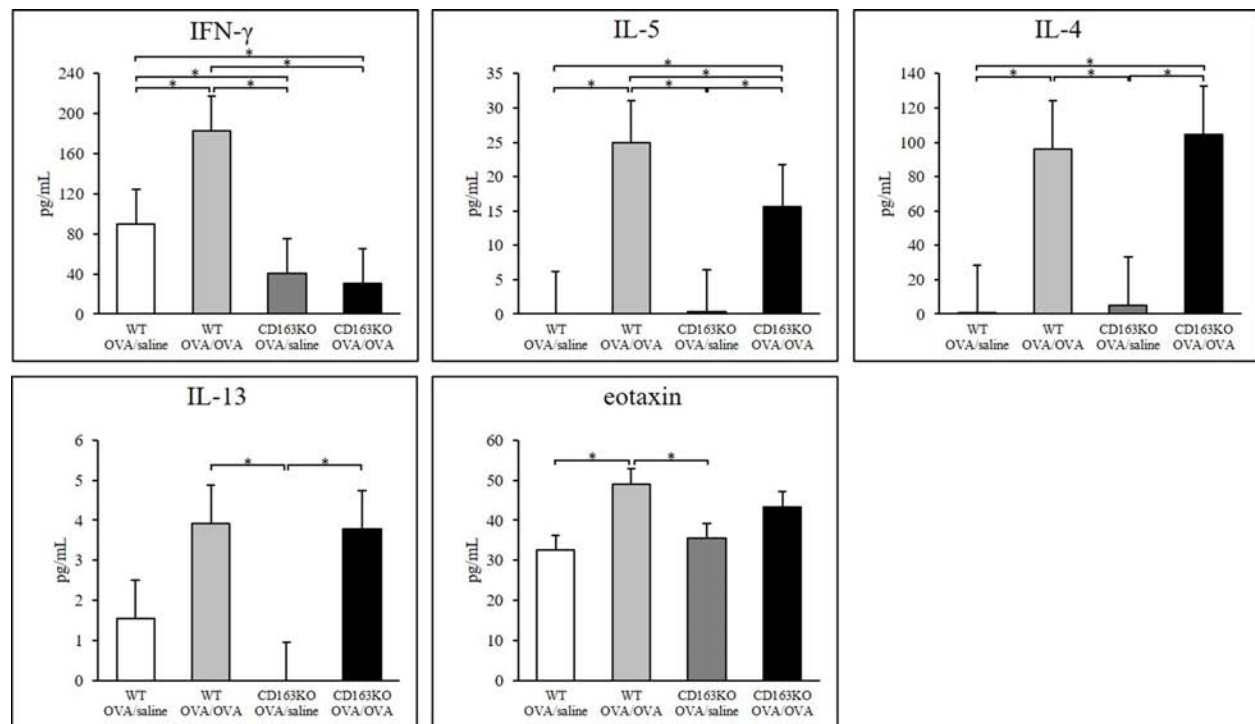












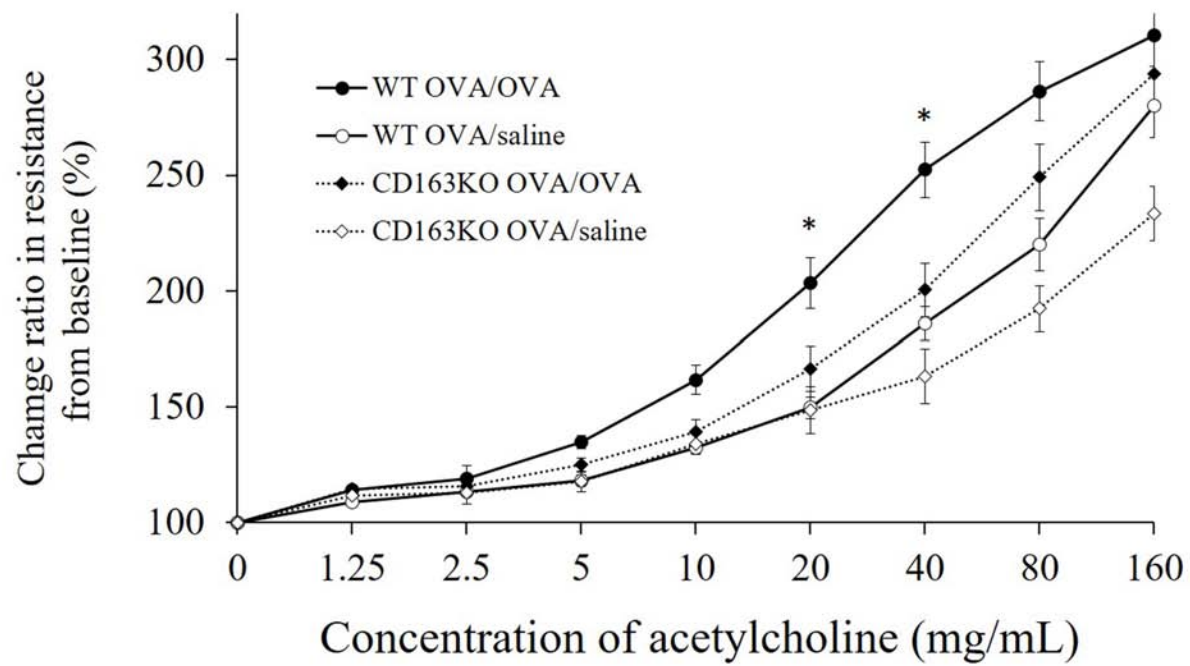


Figure Legends

Figure 1. Study design for a mouse asthma model.

Ovalbumin (OVA)-sensitized mice injected intraperitoneally with OVA on days 0 and 5 and saline-challenged (Group 1) or OVA-challenged (Group 2) on day 18, as reported previously⁸.

Figure 2. Histological analysis of the lung tissues collected from patients who died of asthma.

(A) Immunostaining of the lung tissue samples with CD68 and CD163 from control subjects who were nonsmoker and patients who died of asthma (left: $\times 400$ and right: $\times 40$). Scale bar = 20 μm in the panel left and 100 μm in the right.

(B) The numbers of CD68- and CD163-positive cells in control nonsmokers and patients who died of asthma. *: $p < 0.05$.

Figure 3. Immunostaining of lung tissue samples with Ibal and CD163 from OVA-sensitized and saline-challenged (OVA/saline-) WT mice and OVA-sensitized and OVA-challenged (OVA/OVA-) WT mice (left: $\times 400$ and right: $\times 40$). Scale bar = 20 μm in the left panel and 100 μm in the right panel. Arrows indicated each positive cell

Figure 4. Airway inflammation in the CD163 KO mice mouse asthma model.

(A) Histological tissues of airway inflammation in OVA-sensitized mice. ($\times 200$)

Scale bar = 50 μm . Arrows indicated peribronchial proliferation of inflammatory cells. (HE staining)

(B) Histological tissues of mucous cell metaplasia in OVA-sensitized mice (×400).

Scale bar = 20 μ m. Arrows indicated mucous cell metaplasia. (HE and AB-PAS staining)

(C) Eosinophils in BALFs decreased in CD163KO mice in a mouse asthma model.

(D) The number of total cell, eosinophils, Lymphocyte, Neutrophil, and Macrophages in BALFs decreased in CD 163 KO mice in a mouse asthma model. The cell populations in the BALFs (n = 15–18 per each group) *: p < 0.05

Figure 5. IFN- γ and IL-5 in the BALFs decreased in CD 163 KO mice in a mouse asthma model.

The concentrations of IFN- γ , IL-5, IL-4, IL-13, and eotaxin in the BALFs were measured by specific ELISA kits (n = 15–18 per each group) *: p < 0.05

Figure 6. Airway hyper-responsiveness

The data were expressed as airway resistance changes from the baseline in response to 8 different doses of Ach (n = 10–12 per each group), as described previously^{17, 18}. *: p < 0.05

Acknowledgments

We thank Mr. Takenobu Nakagawa (Kumamoto University), Ms. Emiko Kuma, Ms. Chitoshi Ohki, and Ms. Kyoko Yamaguchi (Kurume University) for their technical assistance. The authors would like to thank Enago (www.enago.jp) for the English language review.